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14. ABSTRACT

Shear forces inflicted by explosion or head impact may result in traumatic retinopathy due to damage of retinal pigment epithelium (RPE) and photoreceptors, leading to loss of sight. Similar injury to photoreceptors and RPE can be induced by exposure to a short pulse laser. Currently there is no therapy for such blind spots (scotomata), and the loss of sight is permanent. This study is focused on development of the animal model of traumatic retinopathy and evaluation of the role of constructive retinal plasticity in elimination or reduction of retinal scotomata and scarring. We established a model of traumatic retinopathy using selective laser coagulation of RPE and photoreceptors in rabbits, based on rapid scanning with a continuous laser. This approach allows creation of the very uniform damage to RPE and photoreceptors over wide areas, sparing the inner retinal neurons in any pigmented animal. We explored the extent of migration of the RPE and photoreceptors from the adjacent non-damaged areas into the damage zone and rewiring of the migrating photoreceptors to local inner retinal neurons. The shift of the photoreceptors into the damage zone over time was monitored with optical coherence tomography and histology. The extent of rewiring was assessed using electrophysiology on a multielectrode array. Within the 4 months we observed complete recovery of the 100 and 200um-wide lines of damage. However, larger damage zones (>400um) contracted only partially.

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Introduction

Shear forces inflicted by explosion or head impact may result in traumatic retinopathy due to damage of the retinal pigment epithelium (RPE) and photoreceptors, leading to loss of sight[1]. Similar injury to photoreceptors and RPE can be induced by exposure to a short pulse laser. Currently there is no therapy for such blind spots (scotomata), and the loss of sight is permanent. This study is focused on development of the animal model of such injury, and on strategies for elimination or reduction of retinal scotomata and scarring based utilization of retinal plasticity. We established a model of traumatic retinopathy using selective laser coagulation of RPE and photoreceptors in pigmented rabbits, based on rapid scanning with a continuous laser. We explore the extent of migration of the photoreceptors from the adjacent non-damaged areas into the damage zone and rewiring of the migrating photoreceptors to local inner retinal neurons[2]. The shift of the photoreceptors into the damage zone over time is monitored with optical coherence tomography and histology. The extent of rewiring is assessed using electrophysiology on a multielectrode array.

Results

The results of our research are presented below, listed by specific tasks:

1) Establish and characterize a model of traumatic retinopathy by creating large scotomata using selective laser coagulation of RPE and photoreceptors in pigmented rabbits. This model will create retinal condition similar to the traumatic retinopathy or retinal dystrophy, including the areas void of photoreceptors, while preserving the inner retinal neurons – inner nuclear layer and ganglion cell layer.

Creating a uniform area of selective damage to RPE and photoreceptors with conventional spot coagulation turned out to be rather challenging since the center of each spot typically exhibits deeper damage than its periphery. To overcome this difficulty and create large uniform and reproducible areas of selective damage to RPE and photoreceptors we developed a new methodology based on rapid scanning of a continuous laser. In this approach our proprietary software directly controls the scanner of a commercial pattern scanning laser[3] (PASCAL, Topcon Medical Laser Systems). By adjusting the power, spot size and scanning speed one can easily control the extent of the retinal damage. By placing lines adjacent to each other one can create large areas of uniform and reproducible damage. To further ensure that no photoreceptors survive at the edges of the line scan, we made the lines overlap by a quarter of the line width. Optimal settings for damaging only the RPE and sparing the photoreceptors was found to be: power 2W, scanning velocity 6 m/s, spot size 100μm. To produce acute damage to photoreceptors, but spare the inner retina, the scanning velocity should be reduced to 1.6 m/s.

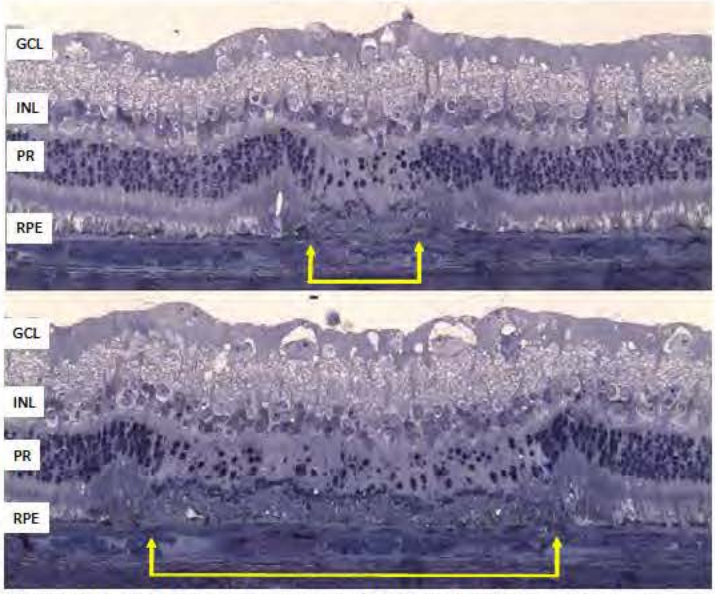

Figure 1. Selective damage to RPE and photoreceptors produced in the rabbit retina by line scanning, as seen 2 days after the treatment. Top: a single scan with 100 μm spot size. Bottom: 4 adjacent scans using the same spot size.

Examples of the selective retinal damage produced by a single scan with

100 μm spot diameter and by 4 adjacent lines of the same spot size are shown in Figure 1. As one can see in these images, the damage is limited to RPE and photoreceptors, sparing the inner retina (INL and GCL) in both lesions, despite the very significant difference in the lesion width. One can resolve 4 "waves" of the coagulated outer segments of the photoreceptors in the wider lesion (bottom image in Figure 1).

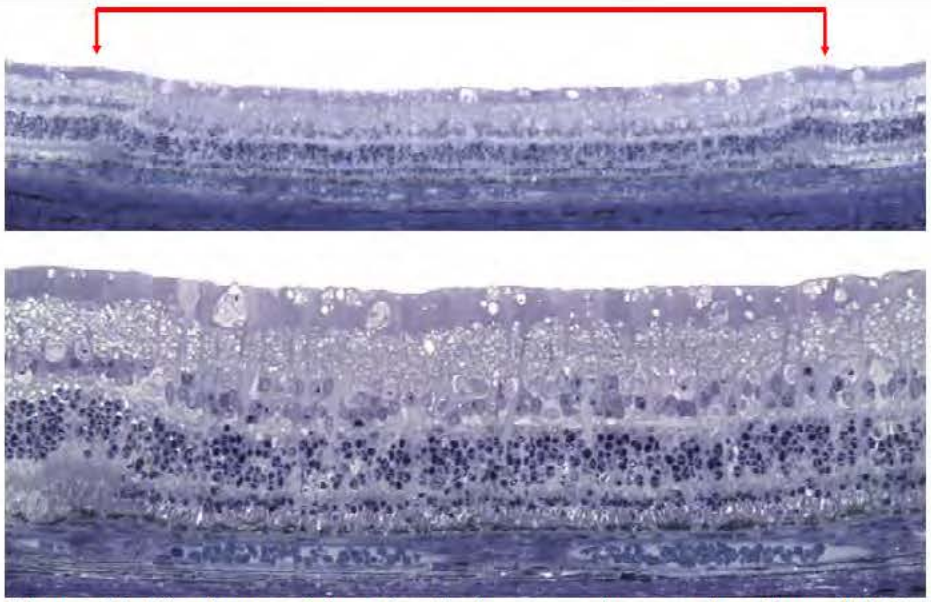

Figure 2A. Histology at 1 day after the laser scanning reveals collapsed RPE and beginning of the damage to photoreceptors over a 1mm-wide area.

Figure 2 shows evolution of the damage to photoreceptors over time after the laser treatment. Damage was produced by scanning over a 1mm-wide area, indicated by the red arrow in the top image in Figure 2A. The lower frame in that Figure shows a higher magnification view of the left edge of the damage zone.

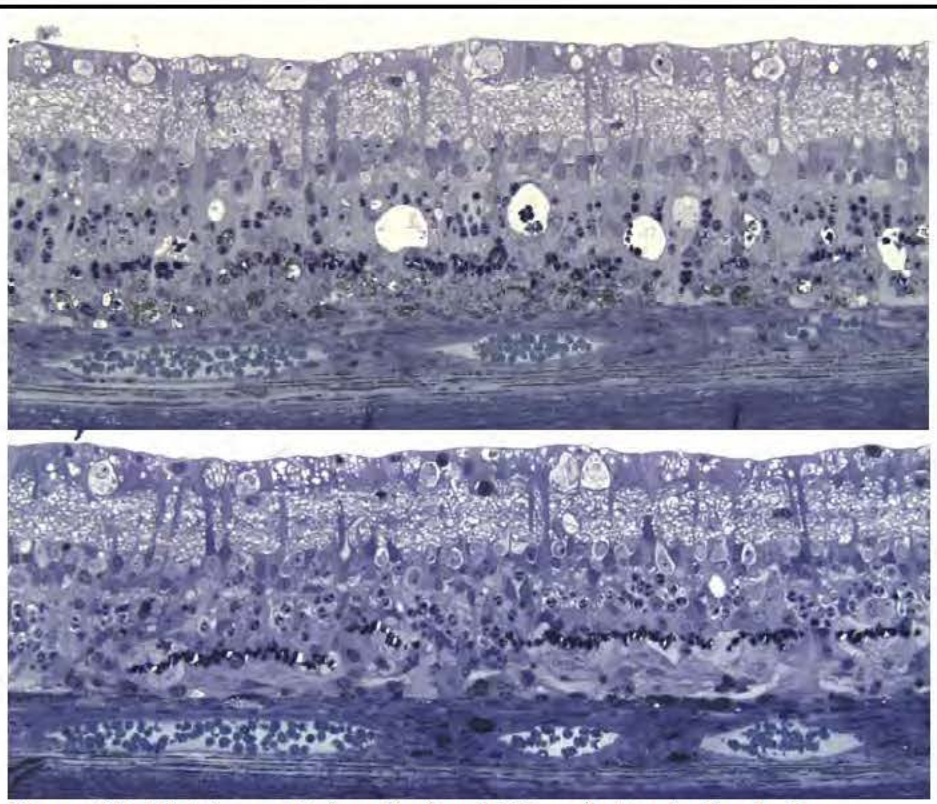

Figure 2B. Histology at 3 days (top) and 7 days (bottom) after the laser scanning demonstrates uniform destruction of the RPE and photoreceptors, with complete preservation of the INL and GCL.

Figure 2B illustrates the process of the photoreceptors disappearance from the damage zone at the later time points. Top image corresponds to the 3 day time point, and the bottom – to 7 days. Despite the complete obliteration of the RPE and photoreceptors over such a wide zone, the inner retina is well-preserved, with no signs of damage to the inner nuclear layer, nor to the ganglion cell layer.

At much lower velocity (0.1 m/s), scanning laser allows creating much more severe retinal damage, including choroidal rupture and bleeding, as illustrated in the Figure 3. Since the goal of this project is selective damage to photoreceptors and RPE, we focused our research on high speed scanning regime (>1 m/s), illustrated in Figures 1 and 2.

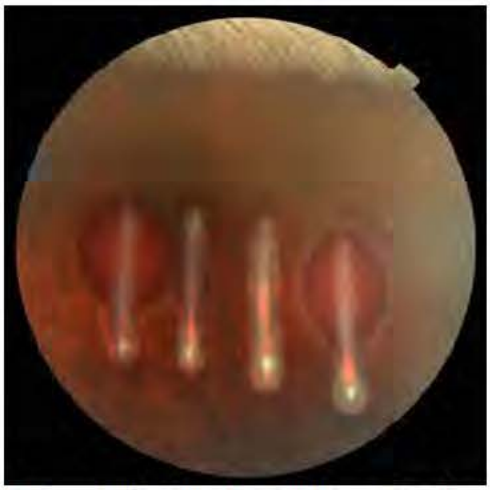

Figure 3. Fundus photo 1 hour after the treatment illustrates severe retinal damage and choroidal bleeding produced by line scanning with 100 μm spot size, 2W power and 0.1 m/s scanning speed.

2) Apply selective laser treatment to shift photoreceptors from the adjacent non-damaged areas into the scotoma. Characterize the effects of the laser exposure parameters on dynamics of the photoreceptors shift and the extent of residual scarring.

As a part of the study of the retinal response to selective destruction of RPE, we evaluated migration and restoration of the RPE monolayer after laser treatment. Figure 4A illustrates destruction of the RPE cells along the line of laser scanning (6 m/s, 2W, 100 μm spot size). RPE cells then stretch and migrate from the undamaged areas, filling the lesion with a few days, as illustrated in Figure 4B. At one month there is no difference in appearance of the RPE in the treated and untreated areas (Figure 4C). At one month after the first treatment a second laser exposure was applied over the same area, and with the same parameters. It resulted in a very similar dynamics of the RPE healing, and no signs of damage could be observed one month after the second treatment, as illustrated in Figure 4D. At one month after the second treatment a third laser exposure was applied over the same area, and with the same parameters. Similar dynamics of the RPE healing was observed, with a complete recovery of the RPE

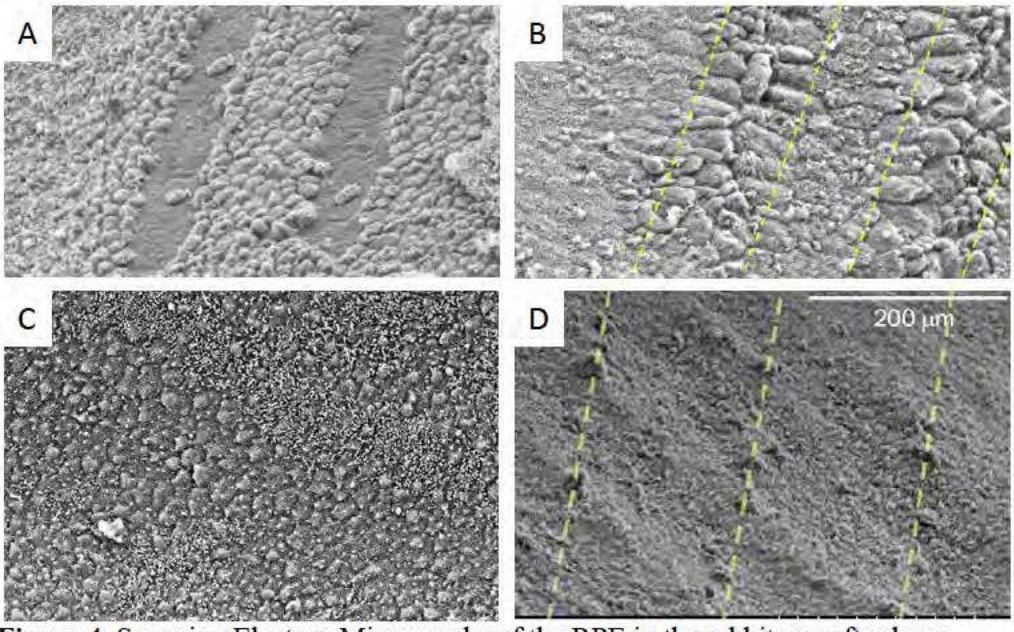

Figure 4. Scanning Electron Micrographs of the RPE in the rabbit eye after laser scanning treatment. A. One day after treatment the RPE cells are destroyed, and lifted with the retina during sample preparation. B. Three days after treatment, RPE cells stretch and shift into the lesions and close the damage zone (between the yellow dsha lines). C. At one month there are no obvious signs of the RPE damage or changes. D. One month after the second treatment RPE completely recovered its monolayer (centers of the scanning lines indicated by the yellow dash lines.

monolayer, without any residual damage observed one months after the 3rd treatment.

Similar dynamics was observed in the RPE lesions of 200 and 400 μm in width. These findings provide reassurance that RPE layer itself can heal rapidly and completely, even after multiple laser treatments over the same area, so the limiting factor in the healing dynamics of the retinal lesions is migration and rewiring of the photoreceptors.

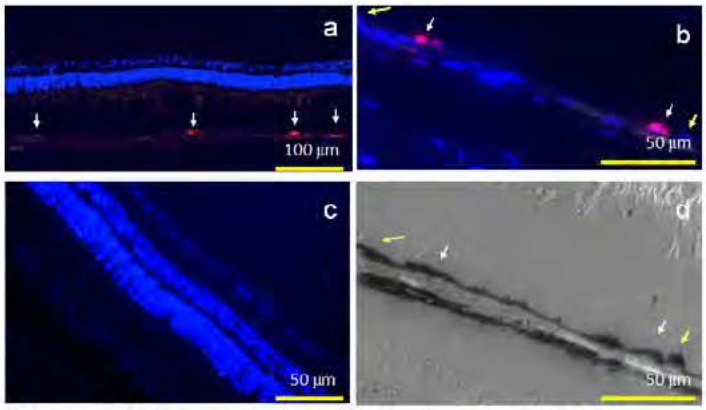

Figure 5. A. Micrographs of BrdU (red) and DAPI (blue) immunostaining of the retina (10x magnification) 4 days after laser treatment. BrdU positive RPE cells are pointed by the white arrows. B. BrdU (red) and DAPI (blue) immunostaining of the retina (40x) 4 days after laser treatment. BrdU positive RPE cells are pointed by the white arrows, BrdU negative cells are pointed by yellow arrows. C. BrdU immunostaining of non-lasered retina from the same animal does not show signs of BrdU uptake. D. Hoffman contrast, white field image of the RPE layer in the same area, as shown in panel b.

To detect the potential proliferation in the RPE and retinal cells, we used 5-bromo-2'-dioxyuridine (BrdU) labeling, which integrates into the DNA of dividing cells. Four eyes from four rabbits received line scanning laser using the same parameters as the treatment protocol of this study four days prior enucleation. BrdU labeling reagent was infused slowly into an ear vein two and one day prior animal sacrifice. Eyes and control samples from small intestines were fixed in formalin, embedded in paraffin, and sectioned into 4-5 μm thick sections. Sections were then incubated with anti-BrdU antibody and Texas Red secondary antibody following instructions of the manufacturer (Invitrogen). At least 5 sections of each sample were analyzed on fluorescent microscopy with mounting media containing DAPI. BrdU immunohistochemistry assay, which labels dividing cells, marked nearly half of the RPE cells in the treated areas (Figure 5). At this time, TEM showed restoration of tight junctions and basal infoldings.

We assessed migration of the photoreceptors and restoration of the retinal function in the damaged areas using electrophysiological recordings from the retina by multielectrode arrays[4]. Signals from the retinal ganglion cells (RGCs) in response to stimulation with spatiotemporal white noise were recorded, and receptive fields of each detected RGC mapped, as illustrated in Figure 6. As expected, laser scanning destroyed photoreceptors and RPE, resulting in an acute scotoma (blind spot), corresponding to the width of the laser scanning zone (100μm, pointed by 2 adjacent arrows). At one month, the width of the scotoma greatly decreased due to migration of the photoreceptors from adjacent areas, leaving very narrow residual gap, as pointed by the single arrows. At 2 months, the scotoma disappeared almost completely, leaving only faint traces of

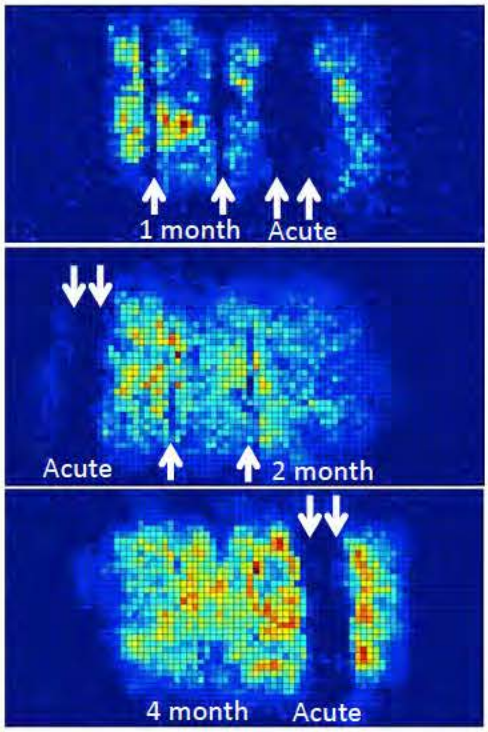

Figure 6. Maps of the retinal sensitivity in the rabbit retina with acute and partially healed 100μm-wide lines of damage at 1, 2 and 4 months.

reduced sensitivity, as can be seen in the middle frame in Figure 6. At 4 months, the scotomata disappeared completely, as shown in the bottom image in Figure 6.

Restoration of the retinal sensitivity in the 200μm wide lesions is illustrated in Figure 7. Acute scotomata correspond to the width of the laser scanning zone (200μm, pointed by 3 adjacent arrows). At one month, the width of the scotoma decreased due to migration of the photoreceptors from adjacent areas, but left more pronounced residual gaps, compare3d to the 100μm lesions (pointed by the single arrows). At 2 months, the scotoma was still visible, as can be seen in the middle frame in Figure 7. At 4 months, the scotomata disappeared completely, as shown in the bottom image in Figure 7.

Areas of the missing photoreceptors in the healing lesions could also be seen using fluorescent marker of rhodopsin, as illustrated in the Figure 4. This Figure illustrates the narrow residual gap (~12μm) in the photoreceptors coverage of the former 100 μm lesion at 1 month. Longer follow-up is required to see further progress of the photoreceptors migration into the lesion.

Feasibility of the photoreceptors migration in human patients has been confirmed by observation of the retinal lesions in human patients using OCT[5]. The follow-up time was limited by 6 months.

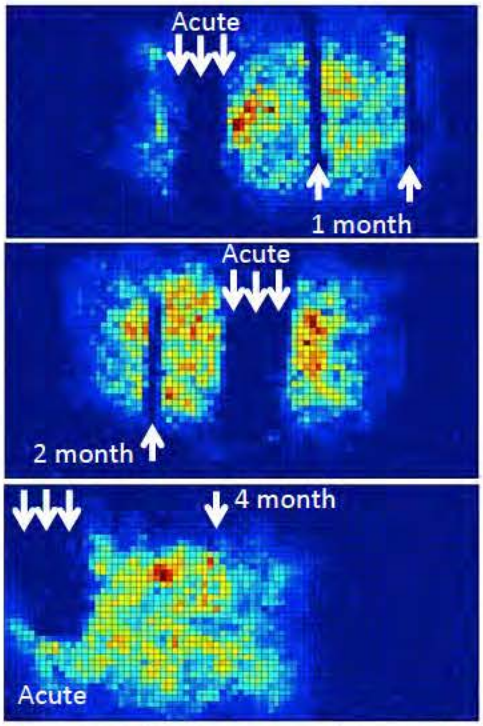

Figure 7. Maps of the retinal sensitivity in the rabbit retina with acute and partially healed 200μm-wide lines of damage at 1, 2 and 4 months.

3) Characterize the effects of repetitive laser treatment for shifting the photoreceptors and RPE cells over extended distances, as a function of the exposure parameters and time delay between the successive treatments.

To assess the effect of repetitive treatments on ability of the RPE cells to migrate and restore the damage zone, we applied three laser scanning procedures to the same areas with one month delay between the treatments. Figure 8a shows fluorescein angiography of the 4 patterns right after the 3rd treatment. After one month fluorescein leakage is absent (Figure 8B), illustrating restoration of the RPE coverage of the area. Light microscopy one month after the third treatment shows no signs of photoreceptor or inner retina damage, but in some areas the RPE cells increased in size, as pointed by the arrow in

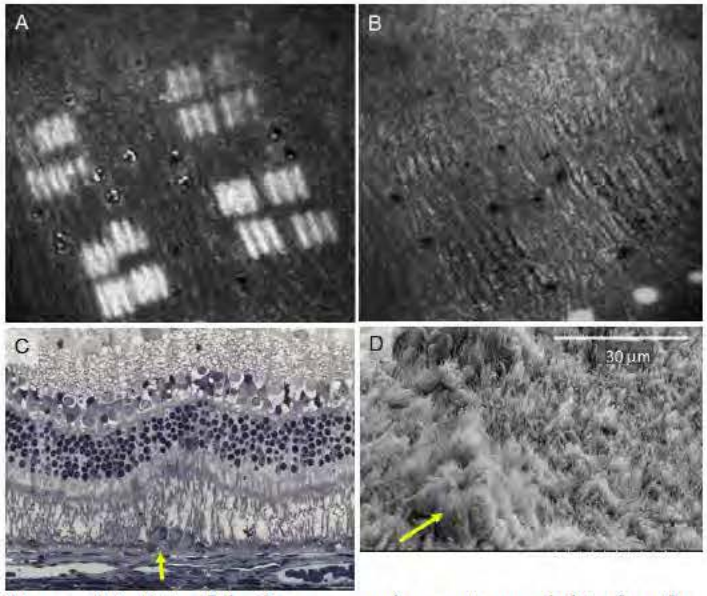

Figure 8A. FA of the laser scanning patterns right after the 3rd treatment. **B**. Same area one month later. **C**. Histology of the retina treated 3 times with line scanning. **D**. SEM of the RPE one month after the 3rd treatment.

Figure 8C. Scanning electron microscopy also shows restoration of the RPE layer, and larger RPE cells in some areas (pointed by the yellow arrow in Figure 8D).

4) *Develop image-guided pattern laser treatment that will apply laser exposures along the perimeter of the damage zone.*

We are working on development of the automated treatment software together with a manufacturer of the PASCAL scanning laser[6] – Topcon Medical Laser Systems Inc. The software package is being developed as a part of the Synthesis Graphic User Interface. The diagnostic image will be displayed on one screen of the system, and the laser treatment parameters on the other. The planned treatment pattern will be then exported into the laser system, and applied relative to the fixation target projected onto the fovea by the aiming beam. Physician will maintain the fixation target on the patient's fovea, and parts of the treatment plan will be applied sequentially upon the press of the foot pedal. Every segment of the treatment pattern is applied within the eye fixation time – no longer than 200ms, so the pattern distortions due to eye motion could be avoided. Figure 9 illustrates a standard version of the Synthesis GUI with a treatment pattern filling a circle with adjustable inner and outer radii, and selectable segments. The pattern is then filled with spots of adjustable diameter and spacing. Laser power is first titrated to the barely visible lesion, and then can be set to any fraction of the titration energy. The EndPoint Management algorithm[7] in the laser software selects the corresponding power and pulse duration from the look-up table produced based on the thermal model or retinal coagulation. The software will also allow for the image guided treatment planning.

Figure 9. Graphic User Interface of the PACAL laser with EndPoint Management algorithm. Segments of the circular pattern are applied sequentially by physician, keeping the aiming beam in the fovea.

Key Research Accomplishments

- We developed a novel animal model of traumatic retinopathy using fast scanning of the retina with a continuous wave laser.
- Rapid scanning with a continuous laser can produce uniform areas of selective damage to the retina layers with adjustable size.
- The extent of the retinal damage can be adjusted by controlling the laser scanning velocity: damage limited to RPE layer (v=6m/s), damage limited to RPE and photoreceptors (v=1-2 m/s), or severe damage to the retina and choroid (v=0.1 m/s).
- RPE migrates and proliferates to fill the damage in the RPE monolayer within days, and completely restores normal anatomy within a month. Such recovery persists after multiple laser treatments of the same area.
- Photoreceptors migrate from the adjacent areas much slower: 100 µm lesion is almost completely recovered within two months, but 200 µm lesion is recovered only partially – by about 70%, and

recovered completely within 4 months.

- Migrating photoreceptors rewire to the surviving local inner retinal neurons, thereby restoring normal retinal anatomy and function.

Reportable Outcomes

Refereed publications:

- Non-genetic Animal Models of Retinal Degeneration. H. Lorach, J. Kung, P. Huie, R. Dalal, Y. Mandel, D. Palanker. *In preparation.*
- Restoration of Retinal Structure and Function after Selective Photocoagulation. A. Sher, B.W. Jones, P. Huie, Y.M. Paulus, D. Lavinsky, L.S. Leung, H. Nomoto, C. Beier, R.E. Marc, and D. Palanker. *The Journal of Neuroscience* **33(16)**: 6800 – 6808 (2013).
- Subvisible Retinal Laser Therapy: Titration Algorithm and Tissue Response. D. Lavinsky, C. Sramek, J. Wang, P. Huie, R. Dalal, Y. Mandel, D. Palanker. *Retina;* **34 (1)**: 87-97 (2014).

Conference presentations:

- Bipolar Cells Restructure Dendrites After Selective Ablation of Photoreceptors. C. Beier, J. Kung, P. Huie, R. Dalal, D. Palanker, A. Sher. ARVO Annual Meeting, Orlando, May 2014.
- Optical Modulation of Transgene Expression in Retinal Pigment Epithelium. D. Palanker, D. Lavinsky, T. Chalberg, Y. Mandel, P. Huie, R. Dalal, M. Marmor. *Ophthalmic Technologies XXI, SPIE*, vol. 8567 (2013).
- Constructive Retinal Plasticity After Selective Ablation of the Photoreceptors Layer. A. Sher, B. Jones, P. Huie, Y. Paulus, D. Lavinsky, L.S. Leung, H. Nomoto, C. Beier, R. Marc, D. Palanker. ARVO Annual Meeting, Seattle, May 2013.

Funding Applied for:

NIH R01 grant # EY023020. Title: Restoration of retinal structure and function after selective photocoagulation of photoreceptors. PI: A. Sher (UCSC), co-investigator: D. Palanker (Stanford U.).

Conclusion

Explosion or head impact may result in traumatic retinopathy due to damage of the retinal pigment epithelium (RPE) and photoreceptors, leading to loss of sight. We developed a strategy for reduction of the retinal scotomata and scarring using retinal plasticity. We established a model of traumatic retinopathy using selective laser coagulation of RPE and photoreceptors in rabbits, based on rapid scanning with a continuous laser. By adjusting the laser scanning speed one can precisely control duration of the exposure in each area, and thereby control the extent of tissue damage. This approach allows creating uniform areas of reproducible retinal damage with adjustable width and depth, mimicking the conditions of traumatic retinopathy in any pigmented animal.

We discovered that RPE migrates and proliferates to fill the damage in the RPE monolayer within days, and completely restores normal anatomy within a month. Such recovery persists after multiple laser treatments of the same area. Photoreceptors migrate from the adjacent areas much slower - within months, and over limited range - about 200μm. Migrating photoreceptors rewire to the surviving local inner retinal neurons, thereby restoring normal retinal anatomy and function.

Retinal treatment software is being developed for automatic computer-guided procedure. The software is based on the PASCAL Synthesis graphic user interface, and it will allow for iage guided treatment planning.

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